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(54) Title: CHIMERIC MOUSE-HUMAN A10 ANTIBODY WITH SPECIFICITY TO A HUMAN TUMOR CELL ANTIGEN

(57) Abstract

A chimeric antibody with human constant region and murine variable region, having specificity to human tumor cells, methods of production, and uses.

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TITLE OF THE INVENTION

CHIMERIC MOUSE-HUMAN A10 ANTIBODY WITH SPECIFICITY TO A HUMAN TUMOR CELL ANTIGEN

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

This invention relates to antibodies, and their derivatives, with specificity for a human tumor antigen, to nucleotide and protein sequences coding therefor, and to methods of obtaining and manipulating such sequences.

BACKGROUND ART

Monoclonal antibody (mAb) technology has greatly impacted current thinking about cancer therapy and diagnosis. The elegant application of cell to cell fusion for the production of mAbs by Kohler and Milstein (Nature (London) 256:495 (1975)) spawned a revolution in biology equal in impact to that of recombinant DNA cloning. MAbs produced from hybridomas are already widely used in clinical studies and basic research, testing their efficacy in the treatment of human diseases including cancer, viral and microbial infections, and other diseases and disorders of the immune system.

Although they display exquisite specificity and can influence the progression of human disease, mouse mAbs, by their very nature, have limitations in their applicability to human medicine. Most obviously, since they are derived from mouse cells, they are recognized as foreign proteins when introduced into humans and elicit immune

responses. Similarly, since they are distinguished from human proteins, they are cleared rapidly from circulation.

Technology to develop mAbs that could circumvent these particular problems has met with a number of obstacles. This is especially true for mAbs directed to human tumor antigens, developed for the diagnosis and treatment of cancer. Since many tumor antigens are not recognized as foreign by the human immune system, they probably lack immunogenicity in man. In contrast, those human tumor antigens that are immunogenic in mice can be used to induce mouse mAbs which, in addition to specificity, may also have therapeutic utility in humans. In addition, most human mAbs obtained <u>in vitro</u> are of the IgM class or isotype. To obtain human mAbs of the IgG isotype, it has been necessary to use complex techniques (e.g. cell sorting) to first identify and then isolate those few cells producing IgG antibodies. A need therefore exists for an efficient way to switch antibody classes at will for any given antibody of a predetermined or desired antigenic specificity.

Chimeric antibody technology, such as that used for the antibodies described in this invention, bridges both the hybridoma and genetic engineering technologies to provide reagents, as well as products derived therefrom, for the treatment and diagnosis of human cancer.

SUMMARY OF THE INVENTION

The invention provides an engineered chimeric antibody of desired variable (V) region specificity and constant (C) region properties, produced after gene cloning and expression of immunoglobulin light (L) and heavy (H) chains. The chimeric antibody and its derivatives which are specific for human tumor antigens may have applicability in the diagnosis and treatment of human cancer. The cloned immunoglobulin gene products and their derivatives can be produced in mammalian or microbial cells.

The invention provides cDNA sequences coding for immunoglobulin heavy and light chains comprising a human C region and a non-human V region.

The invention provides sequences as above, present in recombinant DNA molecules in vehicles such as plasmid vectors, capable of expression in desired prokaryotic or eukaryotic hosts.

The invention provides hosts capable of producing the chimeric antibodies and methods of using these hosts.

The invention also provides individual chimeric immunoglobulin chains, as well as complete assembled molecules having human C regions and mouse V regions with specificity for human tumor cell antigens, wherein both V regions have the same binding specificity.

Among other immunoglobulin chains and/or molecules provided by the invention are:

1. An antibody with monovalent specificity for a human tumor cell antigen, i.e., a complete, functional immunoglobulin molecule comprising:
   (a) two different chimeric H chains, one of which comprises a V region with anti-tumor specificity, and
   (b) two different L chains, with specificity corresponding to the V regions of the H chains. The resulting hetero-bifunctional antibody would exhibit monovalent binding specificity toward human tumor cells antigens.
2. Antibody fragments such as Fab, Fab′, and $F(ab')_2$.

Genetic sequences, especially cDNA sequences, coding for the aforementioned combinations of chimeric chains are also provided herein.

The invention also provides for a genetic sequence, especially a cDNA sequence, coding for the V region of desired specificity of an antibody H and/or L chain, linked to a sequence coding for a polypeptide different from an immunoglobulin chain (e.g., an enzyme). These sequences can be assembled by the methods of the invention, and expressed to yield mixed-function molecules.

The use of cDNA sequences is particularly advantageous over genomic sequences (which contain introns), in that cDNA sequences can be expressed in bacteria or other hosts which lack appropriate RNA splicing systems.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1. Nucleotide sequence of the coding strand for the A10 H chain mouse V region. Shown is the nucleotide sequence from the end of the oligo-dC tail to the J_H2-C_H1 junction. Also shown is the amino acid sequence deduced from the nucleotide sequence. Shown in bold are the oligonucleotides used for site directed mutagenesis and the sites at which restriction site modifications were made.

FIGURE 2. Nucleotide sequence of the coding strand for the A10 mouse kappa V region (V_κ). Shown is the nucleotide sequence from the end of the oligo-dC tail to the $J_\kappa2$-C_κ junction. Also shown is the amino acid sequence deduced from the nucleotide sequence. Shown in bold are the oligonucleotides used for site directed mutagenesis and the sites at which restriction site modifications were made.

FIGURE 3. Construction scheme for the chimeric mouse-human A10 heavy chain mammalian expression plasmid, pING2254. The V region of the cDNA clone pγA10-16 was engineered to be compatible with the eukaryotic expression plasmid pING2227. Plasmid pING2227 contains the following gene regulatory elements useful for expression in mammalian cells: 1) the IgG H chain enhancer element, 2) an Abelson LTR promoter, 3) the SV40 16S splice site, and 4) the IgG H chain polyadenylation signal sequence. It also contains the entire human IgG1 C region from pGMH-6 (Liu, A. Y. et al,, Proc. Natl. Acad. Sci., USA 84:3439 (1987)). pING2227 contains the neomycin phosphotransferase gene which allows for G418 selection in transfected cells.

FIGURE 4. Construction scheme for the chimeric mouse-human A10 light chain mammalian expression plasmid pING2252. The V region from the cDNA clone pKA10-16 was engineered to be compatible with the eukaryotic expression plasmid pING1712. Plasmid pING1712 contains the following gene regulatory elements useful for expression in mammalian cells: 1) the IgH enhancer element, 2) the Abelson LTR promoter, 3) the SV40 16S splice site, and 4) a human κ polyadenylation signal sequence. It also contains the entire human C_κ region (Liu, A.Y., et al. supra) and the GPT gene which allows for mycophenolic acid resistance in transfected cells.

FIGURE 5. Construction scheme for the bacterial chimeric A10 Fab expression plasmid pING3204. Plasmid pING3204 contains the following elements useful for expression in E. coli: 1) the araC gene, 2) the inducible araB promoter, 3) the dicistronic Fd and κ A10 genes fused to the pelB leader sequence, 4) the trpA transcription termination sequence, and 5) the tetR gene, useful for selection in E. coli.

FIGURE 6. Yeast expression plasmids for Fab expression. Shown are the yeast expression plasmids containing the A10 chimeric L chain gene fused to the yeast PGK promoter, invertase signal sequence and PGK polyadenylation signal, a; the similar yeast plasmid contains the Fd gene, b; the yeast expression plasmid containing the L chain promoter/leader fusion with PGK transcription termination signal, c; similar yeast plasmid containing the Fd gene, d; and the final 2 gene yeast expression plasmid pING3203, e.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

GENETIC PROCESSES AND PRODUCTS

The invention provides an antibody that can be used for the treatment and diagnosis of human carcinoma, either alone or in

combination with other reagents. The antigen is that bound by the mAb called A10.

The method of production combines five elements:

1. Isolation of messenger RNA (mRNA) from the mouse B cell hybridoma line producing the mAb, cloning and cDNA production therefrom;

2. Preparation of a full length cDNA library from purified mRNA from which the appropriate V region gene segments of the L and H chain genes can be: (i) identified with appropriate probes, (ii) sequenced, and (iii) made compatible with a C gene segment.

3. Preparation of C region gene segment modules by cDNA preparation and cloning.

4. Construction of complete H or L chain-coding sequences by linkage of the cloned specific immunoglobulin V region gene segments described in 2 above to cloned human C region gene segment modules described in 3.

5. Expression and production of chimeric L and H chains in selected hosts, including prokaryotic and eukaryotic cells.

One common feature of all immunoglobulin L and H chain genes and the encoded messenger RNAs is the so-called J region. H and L chain J regions (J_H and J_L) have different sequences, but a high degree of sequence homology exists (greater than 80%) among each group, especially near the C region. This homology is exploited in this invention wherein consensus sequences of J_H and J_L were used to design oligonucleotides for use as primers or probes for introducing useful restriction sites into the J region for subsequent linkage of V region segments to human C region segments.

C region cDNA module vectors prepared from human cells and modified by site-directed mutagenesis to place a restriction site at the analogous position in the human sequence were used. For example, one can clone the complete human κ chain C region (C_κ) and the complete human gammal C region ($C_\gamma 1$). An alternative method utilizing genomic C region clones as the source for C region module vectors

would not allow these genes to be expressed in hosts such as bacteria where enzymes needed to remove intervening sequences are absent.

Cloned V region segments are excised and ligated to C_L or C_H module vectors. In addition, the human gamma1 region can be modified by introducing a termination codon thereby generating a gene sequence which encodes the H chain portion of an Fab molecule.

The coding sequences with linked V and C regions are then transferred into appropriate expression vehicles for expression in appropriate hosts, prokaryotic or eukaryotic.

Two coding DNA sequences are said to be "operably linked" if the linkage results in a continuously translatable sequence without alteration or interruption of the triplet reading frame. A DNA coding sequence is operably linked to a gene expression element if the linkage results in the proper function of that gene expression element to result in expression of the coding sequence.

Expression vehicles include plasmids or other vectors. Preferred among these are vehicles carrying a functionally complete human C_H or C_L chain sequence having appropriate restriction sites engineered so that any V_H or V_L chain sequence with appropriate cohesive ends can be easily inserted. Human C_H or C_L chain sequence-containing vehicles are thus an important embodiment of the invention. These vehicles can be used as intermediates for the expression of any desired complete H or L chain in any appropriate host.

One preferred host is yeast. Yeast provides substantial advantages for the production of immunoglobulin L and H chains. Yeasts carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies now exist which utilize strong promoter sequences and high copy number plasmids which can be used for production of the desired proteins in yeast. Yeast recognizes leader sequences of cloned mammalian gene products and secretes peptides bearing leader sequences (i.e. prepeptides) (Hitzman, _et al._, _11th International Conference on Yeast, Genetics and Molecular Biology_, Montpellier, France, September 13-17, 1982).

Yeast gene expression systems can be routinely evaluated for the levels of production, secretion and the stability of chimeric H and L chain proteins and assembled chimeric antibodies. Any of a series of yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycolytic enzymes produced in large quantities when yeasts are grown in media rich in glucose can be utilized. Known glycolytic genes can also provide very efficient transcription control signals. For example, the promoter and terminator signals of the iso-1-cytochrome C (CYC-1) gene can be utilized. A number of approaches may be taken for evaluating optimal expression plasmids for the expression of cloned immunoglobulin cDNAs in yeast.

Bacterial strains may also be utilized as transformation hosts for the production of antibody molecules or antibody fragments described by this invention. E. coli strains such as E. coli W3110 (ATCC 27325) and other enterobacteria such as Salmonella typhimurium or Serratia marcescens, and various Pseudomonas species may be used.

Plasmid vectors containing replicon and control sequences which are derived from species compatible with a host cell are used in connection with these bacterial hosts. The vector carries a replication site, as well as specific genes which are capable of providing phenotypic selection in transformed cells. A number of approaches may be taken for evaluating the expression plasmids for the production of chimeric antibodies or antibody chains encoded by the cloned immunoglobulin cDNAs in bacteria.

Other preferred hosts are mammalian cells, grown in vitro or in vivo. Mammalian cells provide post-translational modifications to immunoglobulin protein molecules including leader peptide removal, folding and assembly of H and L chains, glycosylation of the antibody molecules, and secretion of functional antibody protein.

Mammalian cells which may be useful as hosts for the production of antibody proteins include cells of lymphoid origin, such as the hybridoma Sp2/0-Ag14 (ATCC CRL 1581) or the myeloma P3X63Ag8 (ATCC TIB

9), and its derivatives. Others include cells of fibroblast origin, such as Vero (ATCC CRL 81) or CHO- K1 (ATCC CRL 61).

Many vector systems are available for the expression of cloned H chain and L chain genes in mammalian cells. Different approaches can be followed to obtain complete H_2L_2 antibodies. It is possible to co-express H and L chains in the same cells to achieve intracellular association and linkage of H and L chains into complete tetrameric H_2L_2 antibodies. The co-expression can occur by using either the same or different plasmids in the same host. Genes for both H and L chains can be placed into the same plasmid, which is then transfected into cells, thereby selecting directly for cells that express both chains. Alternatively, cells may be transfected first with a plasmid encoding one chain, for example the L chain, followed by transfection of the resulting cell line with a H chain plasmid containing a second selectable marker. Cell lines producing H_2L_2 molecules via either route could be transfected with plasmids encoding additional copies of H, L, or H plus L chains in conjunction with additional selectable markers to generate cell lines with enhanced properties, such as higher production of assembled (H_2L_2) antibody molecules or enhanced stability of the transfected cell lines.

POLYPEPTIDE PRODUCTS

The invention provides "chimeric" immunoglobulin chains, either H or L, with specificity toward human tumor cells. A chimeric chain contains a C region substantially similar to that present in a natural human immunoglobulin, and a V region having the desired anti-tumor specificity of the invention.

The invention also provides immunoglobulin molecules having H and L chains associated so that the overall molecule exhibits the desired binding and recognition properties. Various types of immunoglobulin molecules are provided: monovalent, divalent, or molecules with the invention's V binding domains attached to moieties carrying desired functions.

Various types of immunoglobulin molecules are provided: monovalent, divalent, or molecules with the invention's V region binding domains attached to moieties carrying desired functions. This invention also provides for "fragments" of chimeric immunoglobulin molecules, which include Fab, F(ab'), and $F(ab')_2$ molecules. The invention also provides for "derivatives" of the chimeric immunoglobulins, which term includes those proteins encoded by truncated or modifed genes to yield molecular species functionally resembling the immunoglobulin fragments. The modifications include, but are not limited to, addition of genetic sequences coding for cytotoxic proteins such as plant and bacterial toxins and tumor necrosis factor (TNF). The fragments and derivatives can be produced from any of the hosts of this invention.

Antibodies, fragments or derivatives having chimeric H chains and L chains of the same or different V region binding specificity, can be prepared by appropriate association of the individual polypeptide chains, as taught, for example by Sears, D.W. et al. (Proc. Natl. Acad. Sci. USA 72:353-357 (1975)). With this approach, hosts expressing chimeric H chains (or their derivatives) are separately cultured from hosts expressing chimeric L chains (or their derivatives), and the immunoglobulin chains are separately recovered and then associated. Alternatively, the hosts can be co-cultured and the chains allowed to associate spontaneously in the culture medium, followed by recovery of the assembled immunoglobulin, fragment or derivative.

USES

The antibodies of this invention can be adapted for therapeutic efficacy by virtue of their ability to mediate antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC) against tumor cells. For these activities, either an endogenous source or an exogenous source of effector cells (for ADCC) or complement components (for CDC) can be utilized.

The chimeric antibodies of this invention, their fragments, and derivatives can be used therapeutically as immunoconjugates (see for review: Dillman, R.O., Ann. Int. Med. 111:592-603 (1989)). They can be coupled to cytotoxic proteins, including, but not limited to Ricin-A, Pseudomonas toxin, Diptheria toxin, and TNF. Toxins conjugated to antibodies or other ligands, are known in the art (see, for example, Olsnes, S. et al., Immunol. Today 10:291-295 (1989)). Plant and bacterial toxins typically kill cells by disrupting the protein synthetic machinery.

The chimeric antibodies of this invention, their fragments and derivatives, can be conjugated to additional types of therapeutic moieties including, but not limited to, radionuclides and cytotoxic agents, to treat cancer patients. Examples of radionuclides which can be coupled to antibodies and delivered in vivo to sites of antigen include ^{212}Bi, ^{131}I, ^{186}Re, and ^{90}Y, which list is not intended to be exhaustive. The radionuclides exert their cytotoxic effect by locally irradiating the cells, leading to various intracellular lesions, as is known in the art of radiotherapy.

Cytotoxic drugs which can be conjugated to antibodies and subsequently used for in vivo therapy include, but are not limited to, daunorubicin, doxorubicin, methotrexate, and Mitomycin C. Cytotoxic drugs interfere with critical cellular processes including DNA, RNA, and protein synthesis. For a fuller exposition of these classes of drugs which are known in the art, and their mechanisms of action, see Goodman, A.G., et al., Goodman and Gilman's THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 7th Ed., Macmillan Publishing Co., 1985).

Treatment of an individual with a tumor bearing the antigen recognized by the antibodies, fragments or derivatives of this invention comprises parenterally admininstering a single or multiple doses of the antibody, fragment or derivative. The effective dose is a function of the individual chimeric antibody, the presence and nature of a conjugated therapeutic agent (see above), the patient and his clinical status, and can vary from about 10 ng/kg body weight to about 100 mg/kg body weight. The route of administration may include

intratumoral, intravenous, subcutaneous, intramuscular, intrapulmonary, intraperitoneal, intranasal, intrathecal, transdermal or other known routes.

The chimeric antibodies, fragments or derivatives of this invention may be advantageously utilized in combination with other chimeric antibodies, or with lymphokines or hemopoietic growth factors, etc., which serve to increase the number or activity of effector cells which interact with the antibodies.

The antibodies of the invention having a complete or partial human C region can be introduced in vivo, especially in humans, with reduced negative immune reactions such as serum sickness or anaphylactic shock, as compared to whole mouse antibodies. The antibodies can also be utilized in immunodiagnostic assays in detectably labelled form (e.g., enzymes, ^{125}I, ^{14}C, fluorescent labels, etc.), or in immobilized form (on polymeric tubes, beads, etc.). Such labelled or immobilized antibodies, fragments or derivatives may be provided in kits.

The antibodies, fragments and derivatives may also be utilized in labelled form for in vivo imaging, wherein the label can be a radioactive emitter, a nuclear magnetic resonance contrasting agent (such as a heavy metal nucleus), or an X-ray contrasting agent (such as a heavy metal). The antibodies can also be used for in vitro localization of the recognized tumor cell antigen by appropriate labelling. Mixed antibody-enzyme molecules can be used for immunodiagnosis using assay methods such as ELISA.

Specifically, the chimeric antibodies of this invention can be used for any and all uses in which the murine A10 mAb can be used, with the obvious advantage that the chimeric molecules are more compatible with the human body.

Having now generally described the invention, the same will be further understood by reference to certain specific examples which are included herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

EXAMPLE 1

Preparation of the A10 Murine mAb

1. Preparation of antigen

Thirty μg of carcinoembryonic antigen (CEA) derived from cancer patient ascites was mixed with 120 μg of purified mAb BD6 (Green Cross Corporation, Osaka, Japan), so that the antigenic determinant of a portion of CEA was blocked. The mixture was emulsified with an equal volume of Freund's complete adjuvant and injected into 4 week old male BALB/c mice three times at 10 day intervals. After an additional 10 d. CEA was administered intraperitoneally without Freund's complete adjuvant as a final immunization. Three days after the final immunization, the spleen was removed for cell fusion as described below.

2. Cell fusion and cloning

(a) Fusion

The immune spleen cells and the mouse myeloma, P3U1 (Curr. Top. Microbiol. Immunol., 81:1, 1979), were mixed in the ratio of about 2:1 ($2.2 \times 10^7 : 1.6 \times 10^7$) and a fusion reaction was carried out for 2 min. using 45% polyethylene glycol (average molecular weight: 4000) by a partial modification of the method of Kohler et al. (Immunological Methods, Academic Press, New York, p. 391, 1979). The treated cells were centrifuged, and the supernatant was discarded. The cells were then incubated for five minutes in HAT medium (see below) and inoculated into 96-well flat bottom microplates (0.1 ml per well) and cultured in 7.5% CO_2.

(b) Media used for selection

(1) Dulbecco's modified Eagle medium (DMEM) was obtained from Nissui Seiyaku Co.

(2) HAT medium was prepared by supplementing D-MEM with the following additives: 10% Horse serum (Flow Laboratories); 300 mg/l L-glutamine; 110 mg/l Sodium pyruvate; 100 IU/ml Penicillin; 100 μg/ml Streptomycin; 3.5 g/l Glucose; 3.7 g/l $NaHCO_3$; 1×10^{-4} M Hypoxanthine; 4×10^{-7} M Aminopterin; 1.6×10^{-5} M Thymidine

3. Method of screening

The anti-CEA activity of each hybridoma culture supernatant was screened by the PHA method using sheep red blood cells coupled with CEA. Any wells producing anti-CEA antibodies were further screened by the PHA method using sheep red blood cells coupled with CEA through the anti-MKN-45 mAb, BD6. Selected hybridomas were cloned two or three times and their culture supernatants were checked for reactivity to cancer cell lines.

The hybridoma producing mAb A10 was deposited at the Fermentation Institute of Osaka (IFO) in Osaka, Japan on April 28, 1989 under accession number IFO 50188.

4. Characterization of A10

A10 is an IgG1 that binds to an antigen which is expressed on the surface of cells from many human carcinomas, including colon carcinoma, and is only present at trace levels in normal adult cells.

EXAMPLE 2

A Chimeric Mouse-Human Immunoglobulin with Human Tumor Specificity Produced from Mammalian Cells

1. Recombinant Plasmid and Bacteriophage DNA

Oligo-dG tailed pBR322, pUC18, pUC19, M13mp18, and M13mp19 were purchased from BRL (Gaithersburg, MD). DNA manipulations involving purification of plasmid DNA by buoyant density centrifugation, restriction endonuclease digestion, purification of DNA fragments by agarose gel electrophoresis, ligation and transformation of E. coli were as described by Maniatis, T. et al., Molecular Cloning: A Laboratory Manual, (1982) or other standard procedures. Restriction endonucleases and other DNA/RNA modifying enzymes were purchased from Boehringer-Mannheim (Indianapolis, IN), BRL, and New England Biolabs (Beverly, MA).

2. RNA Purification and cDNA Library Construction

One liter of A10 hybridoma cells at approximately 1 x 10^6 cells/ml were collected by centrifugation and washed in 100 ml of PBS

(8g NaCl, 0.2g KH_2PO_4, 1.15g Na_2HPO_4,and 0.2g KCl per liter). The cells were centrifuged again and the cell pellet was suspended in a solution of guanidine thiocyanate, and total cellular RNA and poly(A)$^+$ RNA were prepared from tissue culture cells by the method described in Maniatis, T. et al., supra.

Oligo-dT primed cDNA libraries were prepared from poly(A)+ RNA by the methods of Gubler, V. et al. (Gene 25:263 (1983)). The cDNA was dC-tailed with terminal deoxynucleotide transferase and annealed to dG-tailed pBR322. cDNA libraries were screened by hybridization (Maniatis, T., supra) with ^{32}P-labelled, nick-translated DNA fragments, ie., for κ clones with a mouse C_κ region probe and for H chain clones with a mouse IgG1 C region probe .

The L and H chain V region fragments from the full length cDNA clones, pKA10-16 and pγA10-16 respectively, were inserted into M13 bacteriophage vectors for nucleotide sequence analysis. The complete nucleotide sequences of the V region of these clones were determined (Figures 1 and 2) by the dideoxy chain termination method. These sequences predict V region amino acid compositions that agree well with the observed compositions, and predict peptide sequences which have been verified by direct amino acid sequencing of portions of the V regions.

The nucleotide sequences of the cDNA clones show that they are immunoglobulin V region clones as they contain amino acid residues diagnostic of V domains (Kabat et al., Sequences of Proteins of Immunological Interest, U.S. Dept. of HHS, 1983). The A10 V_H has the J_H2 sequence and the A10 V_κ has the $J_\kappa 2$ sequence.

3. Construction of Chimeric Expression Plasmids

Expression vectors appropriate for the insertion of V_H and V_L gene modules to obtain expression of chimeric A10 were constructed. The L chain vector pING1712 was made by first making a plasmid DNA containing a test chimeric L chain gene (pING2122) and adding a mouse Abelson LTR promoter, a splice region, and a mouse genomic κ region 3' to the polyadenylation signal. The H chain mouse enhancer 0.7kb XbaI

to EcoRI fragment from M13 M8alphaRX12 (Robinson, R.R. et al. PCT US86/02269) was inserted into XbaI plus EcoRI cut M13mp19. The enhancer-containing HindIII to BglII fragment was inserted into the BglII to HindIII region of pSH6, an E. coli recombinant plasmid DNA that contains unique XhoI, BglII, and HindIII sites, in that order. The enhancer-containing XbaI to XhoI fragment was then inserted into the enhancer XbaI to XhoI region of pING2121b, an expression plasmid identical to pING2108b (Liu, A.Y. et al., J. Immunology 139:3521 (1987)) except that the L6 V_L region (Liu, A.Y. et al., Proc. Natl. Acad. Sci. USA 84:3439 (1987)) was used in its construction instead of the 2H7 V_L region. The resulting plasmid was pING2122.

The mouse Abelson virus LTR was obtained from pelin2 (provided by Dr. Owen Witte, UCLA). pelin2 contains the p120 viral 3′ LTR (Reddy, E.P. et al., Proc. Natl. Acad. Sci. USA 80:3623 (1983)) except that the BglII site at viral position 4623 has been modified by insertion of the EcoRI oligonucleotide linker GGAATTCC. The 0.8kb EcoRI to KpnI fragment of pelin2 containing the p120 3′ LTR promoter was inserted into KpnI plus EcoRI cut pUC18. The LTR was excised as an EcoRI to SalI fragment and ligated to EcoRI plus SalI cut pING2122, resulting in a plasmid where the LTR promoter is adjacent to the L6 L chain gene (pING2126). An XhoI to SalI fragment containing SV40 16S splice donor and acceptor sites was excised from pUC12/pL1 (Robinson, et al., supra) and inserted into the SalI site of pING2126, screening for the orientation where the splice donor was between the LTR and the L chain gene (pING2133).

The polyadenylation/transcription termination region of the κ expression vector was also modified. The first step was the HindIII digestion and religation of plasmid pING2121a, which is identical to pING2108a (Liu, A.Y. et al., supra) except that the L6 V_L was used in its construction instead of the 2H7 V_L, to form pING2121a-deltaH. The 1.1kb BglII to BamHI fragment of mouse genomic DNA distal to the polyadenylation site (Xu, M. et al., J. Biol. Chem. 261: 3838 (1986) was isolated from pS107A (provided by Dr. Randolph Wall, UCLA) and inserted into the BamHI site of pING2121a-deltaH, screening for the

orientation homologous to the native gene. The 3.3kb BglII to SstI fragment containing this modified 3′ region was ligated to the 5.2kb BglII to SstI fragment of pING2121b to form pING1703. The BglII to SalI fragment of pING1703 with the modified 3′ region and chimeric κ coding sequence was ligated to the large BglII to SalI fragment of pING2133, resulting in the 9.1kb κ expression vector pING1712 shown in Figure 4.

The Abelson LTR promoter was also used in the chimeric H chain expression vector pING1714. pING2111 (Robinson, R.R. et al., supra) was modified by the insertion of an AatII oligonucleotide linker at the XbaI site, followed by AatII cleavage and religation to form pING1707. The AatII to SalI fragment containing the Abelson LTR promoter was excised from pING2133 and ligated to the large AatII to SalI fragment of pING1707 to form pING1711. The H chain enhancer was deleted from pING1711 by EcoRI digestion, T4 polymerase treatment, ligation to AatII oligonucleotide linker, and cleavage and religation with AatII to form the 7.7kb expression vector pING1714.

A similar plasmid, pING2227, contains two additional regulatory elements, the IgH enhancer and the human genomic IgG polyadenylation sequence. pING2227 is identical to pING1712 in the region from BglII to SalI containing the IgH enhancer the Abelson LTR promoter, and the 16S splice donor and acceptor sites. The human genomic IgG 3′ end sequence was ligated as an 1185 bp XmaIII DNA fragment into an XmaIII site located 6 bp past the termination codon for the H chain gene in pING1714. The 1300 bp XmaIII fragment containing the genomic gamma 3′ end was isolated from a derivative of pHG3A (Ellison et al. 1982. Nuc. Acids Res. 10: 4071).

4. Construction of a A10 H and L chain Expression Plasmid

The cDNA clone containing the A10 H chain, pγA10-16, was adapted for mammalian expression by introducing convenient restriction endonucleases sites by site directed mutagenesis (Kramer, W. et al., Nuc. Acids Res. 12:9441) into appropriate M13 subclones, Figure 3. Oligonucleotides were synthesized on a Cyclone DNA synthesizer (New Bruns-

wick Scientific Co.) and purified by acrylamide gel electrophoresis. The J-region mutagenesis primer 5'-CTGAGGAGACGGTGACCGTGGTGCCTT-3' was used to insert a BstEII site into the M13 subclone pTK1 generating pTK3, and the oligonucleotide 5'-ATGTCTGTGTCGACCACTGAAGAGA-3' was used to insert a SalI restriction site into pTK3 upstream of the initiation codon ATG, generating pTK7. The restriction fragment containing the A10 V_H region bounded by SalI and BstEII was then cloned into pING2240, a plasmid identical to pING2227 except that it contains a different V_H region to generate the final chimeric A10 H chain vector, pING2254, in two steps as shown in Figure 3.

The cDNA clone containing the A10 L chain, pKA10-16, was adapted for mammalian expression in a similar way, Figure 4. The J-region mutagenesis primer 5'-GTTTTATTTCAAGCTTGGTCC-3' was used to insert a HindIII site into the M13 subclone pTK2 to generate pTK4, and the oligonucleotide 5'-GAACTTTGGTCGACAGCAACGGG-3' was used to insert a SalI restriction site into pTK4 upstream of the initiation codon ATG to generate pTK5. The restriction fragment containing the A10 V_L region bounded by SalI and HindIII was then cloned into the expression vector pING1712 to generate the final chimeric A10 L chain expression vector, pING2252.

5. Stable Transfection of Mouse Lymphoid Cells for the Production of Chimeric Antibody

The cell line Sp2/0 (American Type Culture Collection # CRL1581) was grown in DMEM plus 4.5g/l glucose (GIBCO) plus 10% fetal bovine serum. Media were Supplemented with glutamine, penicillin, and streptomycin (GPS) (Irvine Scientific, Irvine, CA).

The electroporation method of Potter, H. et al. (Proc. Natl. Acad. Sci., USA, 81:7161 (1984)) was used. After transfection, cells were allowed to recover in complete DMEM for 24 hours, and then seeded at 10,000 to 50,000 cells per well in 96-well culture plates in the presence of selective medium. G418 (GIBCO) selection was at 0.8 mg/ml, and mycophenolic acid (Calbiochem) was at 6 µg/ml plus 0.25

mg/ml xanthine. The electroporation technique gave a transfection frequency of 1-10 x 10^{-5} for the Sp2/0 cells.

The chimeric A10 L chain expression plasmid, pING2252, was linearized by digestion with PvuI restriction endonuclease and transfected into Sp2/0 cells, giving mycophenolic acid resistant clones which were screened for L chain synthesis. The best producer after outgrowth and subsequent subcloning, was transfected with PvuI-linearized pING2254, the expression plasmid containing the chimeric A10 H chain gene. After selection with G418, the clone producing the most L plus H chain, Sp2/0-2252 3C3.4D2-2254 6C4, secreted antibody at approximately 6 to 7 μg/μl.

6. Purification of Chimeric A10 Antibody Secreted in Tissue Culture

Sp2/0-2252.3C3.4D2-2254.6C4 (C813) cells were grown in culture medium HB101 (Hana Biologics) + 1% Fetal Bovine Serum, supplemented with 10mM HEPES, 1x GPS (Irvine Scientific #9316). The spent medium was centrifuged at about 14,000 x g for 20 minutes and the supernatant was filtered through a .45 μ Millipore nitrocellulose membrane filter and stored frozen. The antibody level was measured by ELISA. Approximately 17L of cell culture supernatant was concentrated 10-fold over a S10Y30 cartridge using DC-10 concentrator (Amicon Corp). Supernatant containing about 40 mg of chimeric antibody was loaded several times onto a 2 ml Protein A-sepharose column (Pharmacia or equivalent) in PBS, pH 7.4. Elution was performed with a pH step gradient (pH 5.6, 3.5, 2.2), and the chimeric A10 antibody eluted at pH 3.5. Fractions containing antibody (70% yield) were pooled and buffer-exchanged with 10 mM MES, pH 6.0, and loaded several times onto a 1 ml Mono-S FPLC column (Pharmacia). Elution was performed with an NaCl gradient (0-300 mM), and the chimeric A10 antibody eluted at 120 mM NaCl. Fractions containing antibody (22% yield) were pooled and concentrated 80-fold by ultrafiltration (Centricon 30, Amicon Corp.), diluted 5-fold with PBS, reconcentrated 2-fold and finally diluted 16-fold with PBS. The antibody was stored in 1 ml aliquots at -20°.

7. Studies Performed on the Chimeric A10 Antibody

(a) Inhibition of Binding: The mouse A10 mAb and chimeric A10 antibody were compared in a binding inhibition assay. Such inhibition assays are used to establish antigen recognition. Mouse A10 mAb was labeled with biotin; purified unlabeled chimeric A10 and mouse A10 antibodies were examined for their ability to inhibit the binding of labeled A10 antibody to target cells (LS174T colon tumor). The chimeric A10 and mouse A10 antibodies were identical in inhibition of the binding of labeled A10 antibody to LS174T tumor cells (Table 1).

(b) Functional Assays: A comparison was made between the ability of the chimeric A10 and the mouse A10 antibodies to lyse human tumor cells in the presence of human peripheral blood leukocytes as effector cells (mediating ADCC), or human serum as complement (mediating Complement-Dependent Cytolysis, CDC). Table 2 shows that neither the A10 nor chimeric A10 antibody is capable of mediating ADCC. Likewise, neither mouse nor chimeric A10 are able to detectably lyse target LS174T cells in the presence of human serum.

Table 1. Inhibition of Binding of A10 Antibody to LS174T Tumor Cells[a]

	Relative Inhibition by Competing Antibody (A_{490})		
Antibody Conc. (µg/ml)	Chimeric A10	Mouse A10	Human IgG[b]
0.005	.918	.786	.719
0.015	.977	.831	.784
0.046	1.049	.854	.764
0.14	.970	.784	.758
0.41	.851	.778	.726
1.24	.681	.614	.787
3.70	.477	.444	.797
11.11	.354	.316	.776
33.33	.246	.242	.737
100.0	.241	.210	.707
300.0	.153	.151	.688

[a] Biotinylated mouse A10 antibody was incubated with LS174T tumor cells in the presence of the competing antibody at 4°C. Cells were washed free of unbound antibody, and then incubated with avidin peroxidase. A fall in the absorbance at 490 nm (A_{490}) was used as a measure of relative inhibition of binding by biotinylated A10.

[b] Human IgG is used as a nonspecific antibody control.

Table 2. Lack of Antibody-Dependent Cellular Cytotoxicity Mediated by Chimeric A10 Antibody[a]

	% Cytolysis Mediated by:	
Antibody Conc (μg/ml)	Chimeric A10	Mouse A10
50.	33	30
5.	32	26
.5	24	27
.05	27	28
.005	27	28
.0005	27	28
0	29	29

a LS174T tumor cells were labeled with 51Cr, washed, and incubated with freshly isolated peripheral blood leukocytes in the presence of 17% human serum at a ratio of 50 leukocytes per tumor cell for 4 hours at 37°C. The amount of ^{51}Cr released into the medium was used to calculate the % cytolysis as compared to cells lysed by the addition of 1% NP40.

EXAMPLE 3

A Chimeric Mouse-Human Fab with Human Tumor Cell Specificity Produced in Escherichia coli

Bacteria are suited for production of chimeric antibodies expressed from mammalian cDNA since entire coding sequences can be expressed from well characterized promoters. E. coli is one of many useful bacterial species for production of foreign proteins (Holland et al., BioTechnology 4:427 (1986)) since a wealth of genetic information is available for optimization of its gene expression. E. coli can be used for production of foreign proteins internally or for secretion of proteins out of the cytoplasm, where they most often accumulate in the periplasmic space (Gray et al., Gene 39:247 (1985); Oka et al., Proc. Natl. Acad. Sci. USA 82: 7212 (1985)). Secretion from the E. coli cytoplasm has been observed for many proteins and requires a signal sequence. Proteins produced internally in bacteria are often not folded properly (Schoner et al., BioTechnology 3: 151 (1985)). Protein secreted from bacteria, however, is often folded properly and assumes native secondary and tertiary structures (Hsiung et al., BioTechnology 4: 991 (1986).

A Fab molecule consists of two nonidentical protein chains, the intact antibody L chain and the V, J, and C_H1 portions of the antibody H chain (Fd), linked by a single disulfide bridge. The proper cDNA clones for the A10 chimeric L and Fd genes have already been identified. In this example, these cDNA clones were organized into a single bacterial operon (a dicistronic message) as gene fusions to the pectate lyase (pelB) gene leader sequence from Erwinia carotovora (Lei, et al, J. Bacteriol. 169: 4379 (1987)) and expressed from a strong regulated promoter. The result is a system for the simultaneous expression of two protein chains in E. coli, and the secretion of immunologically active, properly assembled Fab of A10 chimeric antibody.

The following sections detail the secretion of chimeric A10 Fab from E. coli.

1. Assembly of the pelB leader sequence cassette

Erwinia carotovora (EC) codes for several pectate lyases (polygalacturonic acid trans-eliminase) (Lei et al, Gene 35: 63 (1985)). Three pectate lyase genes have been cloned, and the DNA sequence of these genes has been determined. When cloned into E. coli under the control of a strong promoter, the pelB gene is expressed and large quantities of pectate lyase accumulate in the periplasmic space and culture supernatant. The pelB signal sequence functions efficiently in E. coli and was used as a secretion signal for antibody genes in this example. (Other signal sequences would also be useful for this application.) The nucleotide sequence surrounding the signal sequence of the pelB gene is published (Lei, et al.,1987, supra). The pelB signal sequence contains a HaeIII restriction site at amino acid 22, adjacent to the signal peptidase cleavage site: ala-ala. Plasmid pSS1004 (Lei, et al., 1987, supra) containing the pelB gene in pUC8 (Vierra and Messing 1982, Gene 19: 259), was digested with HaeIII and EcoR1. This DNA was ligated with an eight base pair SstI linker to SspI and EcoR1 cut pBR322. The resulting plasmid contained a 300 bp fragment which included the 22 amino acid leader sequence of pelB and about 230 bp of upstream E. caratovora DNA. This plasmid, pING173, contains an insert that upon digestion with Sstl and treatment with T4 DNA polymerase can be ligated directly to a DNA fragment flanked by the first amino acid of a mature coding sequence for any gene to generate a protein fusion containing a functional bacterial leader sequence in frame with the incoming gene. The Sstl to EcoR1 restriction fragment in pING173 was cloned into pUC18 (Yanich-Perron et al., Gene 33: 103 (1985)) to generate pRR175, which contains the pelB leader and adjacent upstream non-coding sequence (including a ribosome binding site) downstream of the lac promoter. Plasmid pING1500, derived from pRR175, contains only the region from the -48 of the pelB

gene to an XhoI site downstream of the pelB leader, and includes the SstI site at the junction.

2. In Vitro Mutagenesis

Site directed in vitro mutagenesis was performed as described by Kramer et al., supra, to introduce an SstI restriction site (see Figure 2) into the A10 κ chain cDNA sequence in pTK4 (see Figure 4) at the junction of the leader peptide and mature coding region with the oligonucleotide primer 5'-GAGAACAATTTGAGCTCTGGACAGT-3', generating pTK6.

A PstI site (see Figure 1) was similarly introduced at the junction of the leader peptide and mature coding region of the A10 H chain cDNA sequence in pTK3 with the oligonucleotide primer 5'-AAGCTTCACTTCTGCAGGGACACCATTT-3', generating pTK11 (Figure 2).

3. Preparation of L chain for Bacterial Expression

The chimeric A10 V_L region containing an SstI restriction site at the signal sequence processing site and a unique HindIII site in the J-region of pTK6 served as the starting point for bacterial expression. The plasmid pTK6 was cut with SstI, treated with T4 polymerase, and digested with SalI, Figure 5A, (the SalI site is located outside of the antibody gene segment in the adjacent M13 multicloning region). The approximately 380 bp fragment containing the V_L region was purified and ligated to pING1500 that was cut with SstI, treated with T4 polymerase, and cut with SalI, Figure 5B, generating pTK8. The plasmid pTK8 containing a pelB::A10 V_L region fusion was sequenced to determine that the proper in-frame fusion was formed. A HindIII fragment from pTK8 containing the pelB leader sequence joined to the A10 V-J region was cloned into pS2D, a plasmid that contained the human C_κ region, generating pTK9, Figure 5C.

4. Preparation of Fd for Bacterial Expression

The intact A10 chimeric Fd gene containing a PstI restriction site at the signal sequence processing site and a BstEII restriction site in the J-region in pTK11 served as the starting point for bacterial expression. The plasmid pTK11 was cut with PstI, treated with T4 polymerase, and digested with BstEII, Figure 5D. The approximately 340 bp fragment containing the Fd V-region was purified and ligated to pING1500 that was cut with SstI, treated with T4 polymerase, and cut with XhoI, Figure 5E, along with the BstEII (partial digest) to XhoI restriction fragment containing the human Fd C region and 3 nucleotides following the termination codon from pF3D, Figure 5F, (the human C_H1 region in pF3D was previously generated by introducing a stop codon in hinge, Robinson, R.R. et al., supra). The resulting plasmid that contained a pelB::A10 Fd fusion was sequenced to determine that the proper in-frame fusion was formed. This plasmid was called pTK13.

5. Multicistronic expression system for L chain and Fd gene

For production of bacterially derived Fab, both the L chain and the Fd need to be produced simultaneously within the cell. Using the plasmids constructed with each of these genes separately, a series of expression vectors were constructed that contain both genes aligned so that transcription from a single promoter will specify both genes. This was done in a way that minimized the noncoding DNA between the two genes. Each gene has a ribosome binding site needed for translation initiation and the identical DNA sequence from -48 to the pelB leader::antibody gene junction.

Plasmid pTK9 was cut with SphI, treated with T4 polymerase, cut with EcoRI, and the vector fragment was purified, Figure 5G. Similarly, pTK13 was cut with XhoI, treated with T4 polymerase, cut with EcoRI and the fragment containing the Fd gene was purified, Figure 5H. These two purified DNA fragments were ligated to produce pTK15, which contained the two A10 chimeric gene fusions linked in close proximity. The two gene cistron was placed under the control of the araB promoter

in pING3104. Plasmid pTK15 was cut with SphI, treated with T4 polymerase, cut with XhoI, and the fragment containing the Fd and κ genes was purified, Figure 5I. This DNA fragment was ligated to the vector fragment from pING3104 that had been cut with EcoRI, treated with T4 polymerase, and cut with XhoI, Figure 5J, generating pING3204. This vector contains all the necessary features for expression of A10 chimeric Fab in *E. coli*.

6. Production of Chimeric A10 Fab in Bacteria

Expression of A10 chimeric Fab from pING3204 in *E. coli* is under the inducible control of the araB promoter. Upon arabinose induction, Fab secreted into the growth medium increases more than 10 fold. Uninduced bacterial colonies harboring pING3204 were phenotypically indistinguishable from *E. coli* harboring pING3104. The strain harboring pING3204 was cultured at 32°C in 10L of minimal medium, supplemented with 1.7% glycerol as the carbon source. Dissolved oxygen was maintained at 20% of saturation by addition of a 5X concentrate of glycerol-supplemented minimal medium. The culture was induced at OD_{600}=50 with 0.05% arabinose for greater than 16 hours in a 14L fermenter (Chemap). Fab was detected in the fermentation broth by ELISA.

About 7 liters of culture supernatant was concentrated to 2 liters using a S10Y10 cartridge (DC10 concentrator, Amicon Corp.). The concentrate is passed through a 500g DEAE cellulose type DE52, Whatman) column pre-equilibrated with 10mM sodium phosphate at pH 8.0. Sufficient 0.2M monosodium phosphate is added to adjust pH to 6.8, and the sample is concentrated over a YM10 membrane (Stirred Cell 2000, Amicon). The sample was then diluted with sufficient water and reconcentrated to 200ml to give a conductivity of 1.1mS/cm. The total amount of protein was estimated by a colorimetric assay, and the sample was loaded onto a carboxymethylcellulose type (CM52, Whatman) column at a ratio of 10mg total protein per 1g CM52 (pre-equilibrated with 10mM sodium phosphate buffer at pH 6.8). The CM52 column was eluted with a linear gradient of increasing NaCl concentration

(0-0.1N) in the same phosphate buffer. The fractions containing Fab (as assessed by ELISA) were further analyzed by SDS-PAGE and then pooled. The combined Fab fractions were concentrated over a YM10 membrane to an Fab concentration of about 1 mg/ml, and stored frozen.

The A10 Fab purified from E. coli has identical molecular weight properties as other Fab molecules produced from E. coli, as assessed by SDS gel electrophoresis. The bacterially-produced A10 Fab is correctly assembled as a κ + Fd chain dimer because of its positive reaction in the ELISA which detects molecules with both κ and Fd determinants, and because it reacts with antigen positive cell lines.

7. Binding Characteristics of Fab Protein Secreted by E. coli

The E. coli A10 Fab was shown to bind to antigen positive cell by performing direct and competition binding assays with the human carcinoma cell line LS174T. In the direct binding assay, Fab bound to the same target cancer cells as did mouse A10 antibody, but not to a control cell line which lacks the antigen. In the competition assay using ^{125}I - labeled mouse A10 antibody, the E. coli-derived chimeric A10 Fab inhibited binding of radiolabeled mouse A10 mAb to the human tumor cell line LS174T. Fifty percent inhibition of A10 mAb binding by the yeast-derived Fab occurred at a concentration of about 1 μg/ml (Table 3), about the same concentration at which A10 mouse mAb showed 50% inhibition. Yeast derived A10 Fab inhibited the binding of mouse A10 antibody and mouse A10 Fab to LS174T tumor cells. THe mouse Fab was prepared by papain digestion of mouse whole 939 antibody.

Table 3. Inhibition of Binding of A10 Antibody to LS174T Tumor Cells

Antibody Conc. (μg/ml)	% Inhibition by Competing Antibody[a]			
	Mouse A10	Chimeric A10 Fab	Mouse A10 Fab[b]	Human IgG[c]
324	100	92	100	15
108	98	99	99	13
36	96	98	99	17
12	90	94	98	18
4	73	76	88	10
1.33	47	57	72	21
0.44	28	39	45	17
0.15	17	30	24	-3
0.049	19	11	9	-15
0.016	20	13	7	-3
0.005	0	3	-1	-9

a ^{125}I-labeled A10 antibody was incubated with LS174T tumor cells in the presence of the competing antibody at 4°C. Cells were washed free of unbound antibody, and cell-bound radioactivity was used to determine the % inhibition of binding.

b Prepared by papain treatment

c Human-IgG was used as a nonspecific antibody control.

EXAMPLE 4

A Chimeric Mouse-Human Fab with Human Tumor Cell Specificity Produced in Yeast

Yeast cells are capable of expressing and secreting foreign proteins. In this example, yeast serve as a host for the production of mouse-human chimeric Fab. This reagent may prove useful in cancer diagnosis by in vivo imaging of appropriately labeled Fab, and in cancer therapy by administration of the Fab as a drug, radionuclide, or toxin immunoconjugate.

1. Yeast Strains and Growth Conditions

Saccharomyces cerevisiae strain PS6 (*ura*3 *leu*2 *MAT*a) was developed at INGENE and used as a host for yeast transformations performed as described by Ito et al., J. Bacteriol. 153: 163-168 (1983). Yeast transformants were selected on SD agar (2% glucose, 0.67% yeast nitrogen base, 2% agar) and grown in SD broth buffered with 50mM sodium succinate, pH5.5.

2. Construction of Yeast Expression Plasmids Containing Antibody Genes

The gene sequences encoding the mature form of the V_L region of A10 and containing a *Hind*III site in the J region and an *Sst*I site introduced at the signal sequence processing site was fused to the human C_κ region. Plasmid pTK6 (Figure 5) was cut with *Sst*I, treated with T4 polymerase, and cut with *Hind*III. The V-region fragment was ligated along with the human C_L region prepared from pM1D (the same fragment as contained in pS2D, see Example 2 and Figure 5) as a *Hind*III to *Xho*I fragment into pING1149. The plasmid pING1149 contains the gene sequence encoding the yeast invertase signal sequence (Taussig, R., et al., Nucl. Acids Res. 11:1943-1954 (1983)) under the control of the yeast PGK promoter (Hitzeman, R.A. et al., Nucl. Acids Res. 10:7791-7800 (1982)).

The resultant plasmid pTK12, Figure 6A, contains the PGK promoter (P) along with the invertase signal sequence (S) fused to the A10 coding sequence. As a result of this fusion, the gene sequence encoding the mature form of the A10 chimeric L chain was fused in frame to the gene sequence encoding the yeast invertase signal sequence. The PGK promoter - invertase signal sequence - chimeric L chain (V,C_{κ}) fusion was cloned into a partial 2 micron circle (2 μ), ura yeast expression vector containing the PGK polyadenylation signal (Tm) to generate pTK16 (Figure 6B).

The gene sequence encoding the mature form of the V_H region of A10 containing a BstEII site in the J region and a PstI site introduced at the signal sequence processing site from pTK11 (Figure 5) was fused along with the human C_H1 region (which had been previously generated by introducing a stop codon in hinge, Robinson, R.R. et al. PCTU586/02269) from pF3D (see Example 2 and Figure 5) into pING1149 that had been cut with PstI, treated with T4 polymerase and cut with XhoI. This generated plasmid pTK14, Figure 6C.

The PGK promoter-invertase signal sequence - chimeric Fd chain (V,C_H1) fusion was cloned into a partial 2 micron circle (2μ) expression vector containing the PGK polyadenylation signal (Tm) to generate pTK17 (Figure 6D).

A single yeast expression vector containing both the chimeric L chain and Fd chain genes and their respective expression signals was constructed from pTK17 and pTK16. This final vector, pING3203, Figure 6E, contains a portion of 2 micron circle (oriY, REP3) and the two selectable markers leu2d and ura3.

3. Production of Chimeric A10 Fab in Yeast

The plasmid pING3203 was transformed into S. cerevisiae PS6 and the transformants were grown in broth under selective conditions as described above. The culture supernatants were assayed by ELISA and contained Fab levels of approximately 100 ng/ml. This material can be purified from this fermentation broth and is expected to have proper-

ties identical to those of Fab produced as described in example 2; yeast A10 Fab is expected to bind to LS174T cells.

CONCLUSIONS

The examples presented above demonstrate a number of important characteristics of the chimeric A10 antibody and the genetically engineered A10 Fab proteins of the invention. First, both the chimeric A10 antibody and its Fab derivative bind to human tumor cell lines to the same extent as the mouse A10 antibody with approximately the same avidity. The chimeric A10 antibody is significant because it binds to the surface of human tumor cells. The A10 mAb has minimal reactivity to normal cells such as fibroblasts, endothelial cells, or epithelial cells in the major organs. Thus, the chimeric A10 mAb defines an antigen that is useful in vitro for detecting and distinguishing human tumor cells.

Although the prospect of attempting tumor therapy using mAbs is attractive, to date such mAb therapy has been met with only limited success (Houghton et al., Proc. Natl. Acad. Sci. 82:1242-1246 (1985)). The therapeutic efficacy of unmodified mouse mAbs appears to be too low for most practical purposes. The chimeric A10 is an improved therapeutic agent over the mouse A10 mAb for the treatment of human tumors in vivo. First, the high biological activity of the chimeric A10 antibody against human tumor cell lines combined with minimal reactivity with normal tissues imply that this antibody may mediate the destruction of malignant tissue. Second, the "more human" chimeric A10 antibody will be more resistant to rapid clearance from the body than the mouse A10 antibody. The human component of a chimeric antibody may enhance its ability to mediate target destruction in combination with human effector cells or complement.
Third, the enhanced resistance to clearance means that chimeric A10 antibodies and their derivatives can be advantageously used for the diagnosis and therapy of tumors in the form of immunoconjugates with drugs, toxins, immunomodulators, radionuclides, etc. Such immunoconjugates, and techniques to form them, are known to those skilled

in the art and can be used to modify the chimeric A10 antibody within the scope of the present invention.

Moreover, using the methods disclosed in the present invention, any desired antibody isotype can be combined with any particular antigen combining site. This invention also enables the direct production of one or more domains of the antibody molecule in functionally active form.

DEPOSITS

Two illustrative cell lines secreting chimeric A10 antibody were deposited prior to the U.S. filing date at the ATCC, Rockville MD.

1. Transfected hybridoma Sp2/0 carrying (pING2252 and pING2254), designated strain C813 (ATCC accession # HB10195)

2. E. coli MC 1061 (pING3204), designated strain G268 (ATCC Accession # 68071)

WHAT IS CLAIMED IS:

1. A polynucleotide molecule comprising a sequence coding for the variable region of an immunoglobulin chain having specificity to the antigen bound by the murine A10 antibody.

2. The molecule of claim 1 wherein said chain is a heavy chain.

3. The molecule of claim 1 wherein said chain is a light chain.

4. The molecule of claim 1 which further comprises an additional sequence coding for at least part of the constant region of a human immunoglobulin chain, both said sequences in operable linkage with each other.

5. The molecule of claim 4 wherein said additional sequence is a cDNA sequence.

6. The molecule of claim 1 which is a recombinant DNA molecule.

7. The molecule of claim 6 which is in double stranded DNA form.

8. The molecule of claim 7 which is an expression vehicle.

9. The molecule of claim 8 wherein said vehicle is a plasmid.

10. A prokaryotic host transformed with the molecule of claim 4.

11. The host of claim 10 which is a bacterium.

12. A eukaryotic host transfected with the molecule of claim 4.

13. The host of claim 12 which is a yeast cell or a mammalian cell.

14. A chimeric immunoglobulin heavy chain comprising at least part of a human constant region and at least part of a variable region having specificity to the antigen bound by the A10 murine monoclonal antibody.

15. A chimeric immunoglobulin light chain comprising at least part of a human constant region and at least part of a variable region having specificity to the antigen bound by the A10 murine monoclonal antibody.

16. A chimeric antibody molecule comprising two light chains and two heavy chains, each of said chains comprising at least part of a human constant region and at least part of a variable region having specificity to the antigen bound by the A10 murine monoclonal antibody, or a fragment or derivative of said chimeric antibody.

17. The antibody, fragment or derivative of claim 16 in detectably labelled form.

18. The antibody, fragment or derivative of claim 17 wherein said label is a radionuclide.

19. The antibody, fragment or derivative of claim 16 conjugated to a cytotoxic drug.

20. The antibody, fragment or derivative of claim 16 conjugated to a cytotoxic protein.

21. The antibody, fragment or derivative of claim 16 immobilized on a solid phase.

22. A process for preparing a chimeric immunoglobulin heavy chain having at least part of a human constant region and at least part of a variable region with specificity to the antigen bound by the A10 murine monoclonal antibody comprising:

(a) culturing a host capable of expressing said heavy chain under culturing conditions,
(b) expressing said heavy chain; and
(c) recovering said heavy chain from said culture.

23. A process for preparing a chimeric immunoglobulin light chain having at least part of a human constant region and at least part of a variable region with specificity to the antigen bound by the A10 murine monoclonal antibody comprising:

(a) culturing a host capable of expressing said light chain under culturing conditions;
(b) expressing said light chain; and
(c) recovering said light chain from said culture .

24. A process for preparing a chimeric immunoglobulin, fragment or derivative containing a heavy chain and a light chain, each of said heavy and light chains having at least part of a human constant region and at least part of a variable region with specificity to the antigen bound by the A10 murine monoclonal antibody comprising:

(a) culturing a host capable of expressing said light chain under culturing conditions, expressing said light chain, and recovering said light chain from said culture;
(b) separately culturing a host capable of expressing said heavy chain under culturing conditions, expressing said heavy chain, and recovering said heavy chain from said culture; and
(c) associating said recovered heavy chain and light chain, thereby preparing said chimeric immunoglobulin, fragment or derivative.

25. A process for preparing a chimeric immunoglobulin, fragment or derivative containing a heavy chain and a light chain, each of said heavy and light chains having at least part of human constant region and at least part of a variable region with specificity to the antigen bound by the A10 murine monoclonal antibody comprising:

(a) co-culturing a host capable of expressing said heavy chain with a host capable of expressing said light chain under culturing conditions;
(b) expressing said heavy chain and said light chain;
(c) permitting said heavy chain and said light chain to associate into said chimeric immunoglobulin, fragment or derivative; and
(d) recovering said chimeric immunoglobulin, fragment or derivative from said culture.

26. A process for preparing a chimeric immunoglobulin, fragment or derivative containing a heavy chain and a light chain, each of said heavy and light chains having at least part of a human constant region and at least part of a variable region with specificity to the antigen bound by the A10 murine monoclonal antibody comprising:

(a) culturing a host capable of expressing said heavy chain and said light chain under culturing conditions;
(b) expressing said chimeric immunoglobulin, fragment or derivative and
(c) recovering from said culture said chimeric immunoglobulin, fragment or derivative

27. The process of any of claims 22-26 wherein said host is prokaryotic.

28. The process of any of claims 22-26 wherein said host is eukaryotic.

29. An immunoassay method for detecting an antigen capable of binding to the A10 murine monoclonal antibody in a sample comprising:

(a) contacting said sample with the antibody, fragment or derivative of claim 16, 17, 18 or 21; and

(b) detecting said antigen by detecting the binding of the antibody, fragment or derivative.

30. An imaging method for detecting an antigen capable of binding to the A10 murine monoclonal antibody in an animal, comprising:

(a) contacting the labelled antibody, fragment or derivative of claim 17 with said animal; and

(b) detecting said antigen.

31. A method of killing cells carrying an antigen thereon, which antigen is capable of binding to the A10 murine monoclonal antibody, comprising:

(a) delivering to said cells the antibody, fragment or derivative of any of claims 16-20; and

(b) allowing said killing to occur.

32. The method of claim 31 wherein said killing occurs by antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity, radiation cytotoxicity, cytotoxic drug action, or protein toxin action.

33. A method of treating a subject suspected of having a tumor bearing an antigen which is capable of binding to the A10 murine antibody comprising administering to said subject an effective dose of the antibody, fragment or derivative of any of claims 16-20.

```
                                                    3'-AGAGAAG
GCCCCTCCATACCCCCCATCCCTTAGACACACAAACCTGGACTCACAAGTTTTTCTCTTC
            15             30             45             60

    SalI
TCACCAGCTGTGTCTGTA-5'
                               METTyrLeuGlyLeuAsnTyrValPheIle
AGTGACAGACACAGACATAGAACATTCACGATGTACTTGGGACTGAACTATGTATTCATA
            75             90            105            120

                       PstI
       3'-TTTACCACAGGGACGTCTTCACTTCGAA-5'
ValPheLeuLeuAsnGlyValGlnSerGluValLysLeuGluGluSerGlyGlyGlyLeu
GTTTTTCTCTTAAATGGTGTCCAGAGTGAAGTGAAGCTTGAGGAGTCTGGAGGAGGCTTG
           135            150            165            180

ValGlnProGlyGlySerMETLysLeuSerCysThrAlaSerGlyPheThrPheSerAsp
GTGCAACCTGGAGGATCCATGAAACTCTCTTGTACTGCCTCTGGATTCACTTTAGTGAC
           195            210            225            240
```

FIG. 1

```
AlaTrpMETAspTrpValArgGlnSerProGluLysGlyLeuGluTrpValAlaGluIle
GCCTGGATGGACTGGGTCCGCCAGTCTCCAGAGAAGGGGCTTGAGTGGGTTGCTGAAATT
          255          270          285          300

ArgSerLysValAsnAsnHisAlaThrTyrTyrAlaGluSerValLysGlyArgPheThr
AGAAGCAAAGTTAATAATCATGCAACATATTATGCTGAGTCTGTGAAAGGGAGGTTCACC
          315          330          345          360

IleSerArgAspAspSerLysSerSerValTyrLeuGlnMETAsnSerLeuGlyThrGlu
ATCTCAAGAGATGATTCCAAAAGTAGTGTCTACCTGCAAATGAACAGTTTAGGAACTGAA
          375          390          405          420

                                                    3'-TTCCGTGG
AspThrGlyIleTyrTyrCysLeuThrGlyPheTyrPheAspTyrTrpGlyGlnGlyThr
GACACTGGCATTTATTACTGTCTAACTGGGTTTTACTTTGACTACTGGGGCCAAGGCACC
          435          450          465          480

  BstEII
TGCCAGTGGCAGAGGAGTC-5'
ThrLeuThrValSerSer
ACTCTCACAGTCTCCTCA
          495
```

FIG. 1(cont.)

```
            SalI
3'-GGGCAACGACAGCTGGTTTCAAG-5'        METAspPheGlnValGlnIlePhe
GCCCCCCGTTGCTAGGGACCAAAGTTCAAAGACAAAATGGATTTTCAAGTGCAGATTTTC
            15             30             45             60

                                       SstI
                         3'-TGACAGGTCTCGAGTTTAACAAGAG-5'
SerPheLeuLeuIleSerAlaSerValIleLeuSerArgGlyGlnIleValLeuThrGln
AGCTTCCTGCTAATCAGTGCCTCAGTCATACTGTCCAGAGGACAAATTGTTCTCACCCAG
            75             90            105            120

SerProAlaIleMETSerAlaSerProGlyGluLysValThrMETThrCysSerAlaSer
TCTCCAGCAATCATGTCTGCATCTCCAGGGGAGAAGGTCACCATGACCTGCAGTGCCAGC
           135            150            165            180

SerSerValSerTyrMETTyrTrpTyrGlnGlnLysProGlySerSerProArgLeuLeu
TCAAGTGTAAGTTACATGTACTGGTACCAGCAGAAGCCAGGATCCTCCCCCAGACTCCTG
           195            210            225            240
```

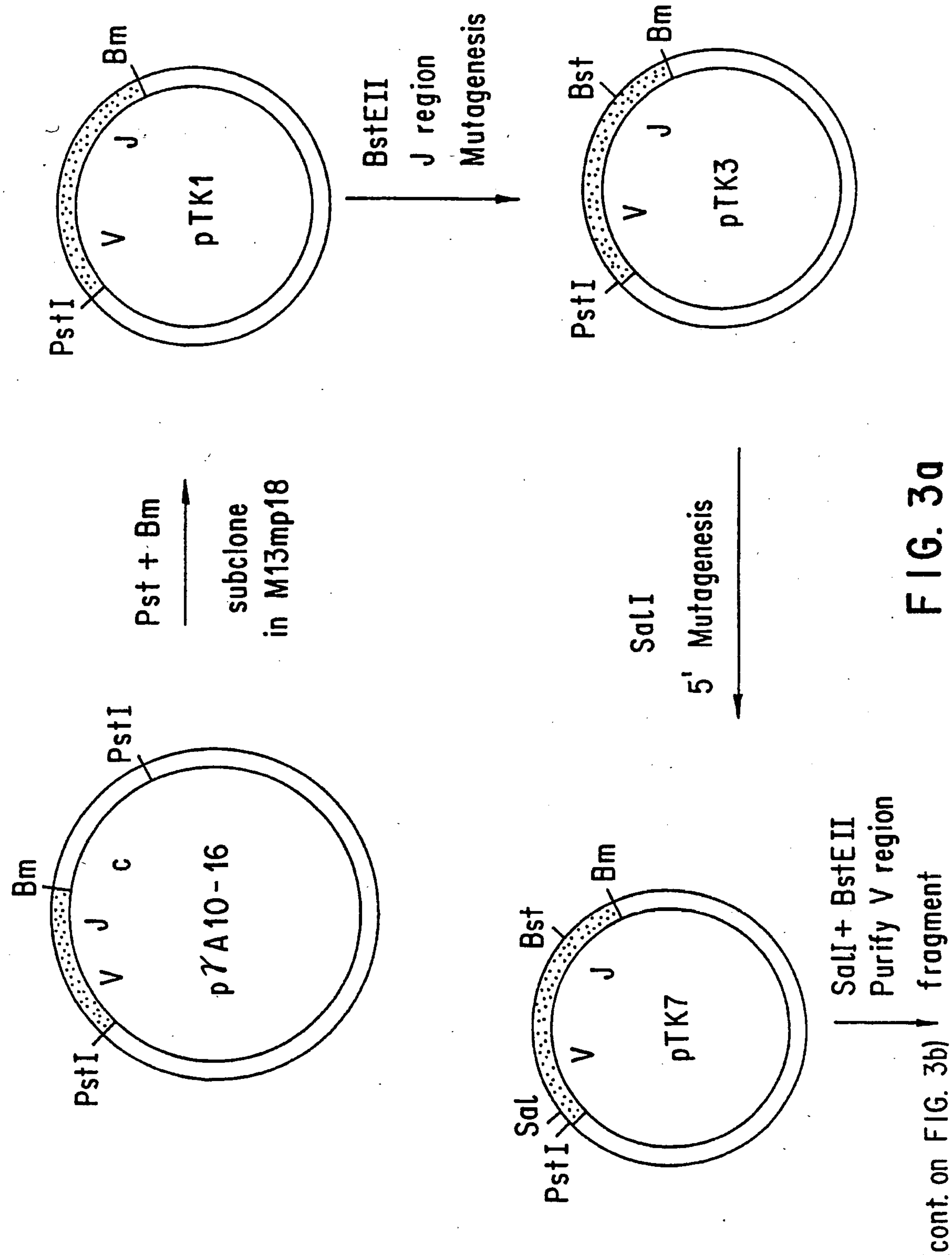
FIG. 2

```
IleTyrGluThrSerAsnLeuAlaSerGlyValProValArgPheSerGlySerGlySer
ATTTATGAGACATCCAACCTGGCTTCTGGAGTCCCTGTTCGCTTCAGTGGCAGTGGGTCG
                255            270            285            300

GlyThrSerTyrSerLeuThrIleSerArgMETGluAlaGluAspAlaAlaThrTyrTyr
GGGACCTCTTACTCTCTCACAATCAGCCGAATGGAGGCAGAAGATGCTGCCACTTATTAC
                315            330            345            360

                                                  HindIII
                                         3'-CCTGGTTCGAACTTTATTTTG-5'
CysGlnGlnTrpSerArgTyrProProThrSerGlyGlyGlyThrLysLeuGluIleLys
TGCCAGCAGTGGAGTCGTTACCCACCCACGTCCGGAGGGGGGACCAAGCTGGAAATAAAA
                375            390            405            420
```

FIG. 2(cont.)



(cont. on FIG. 3b)

FIG. 3a

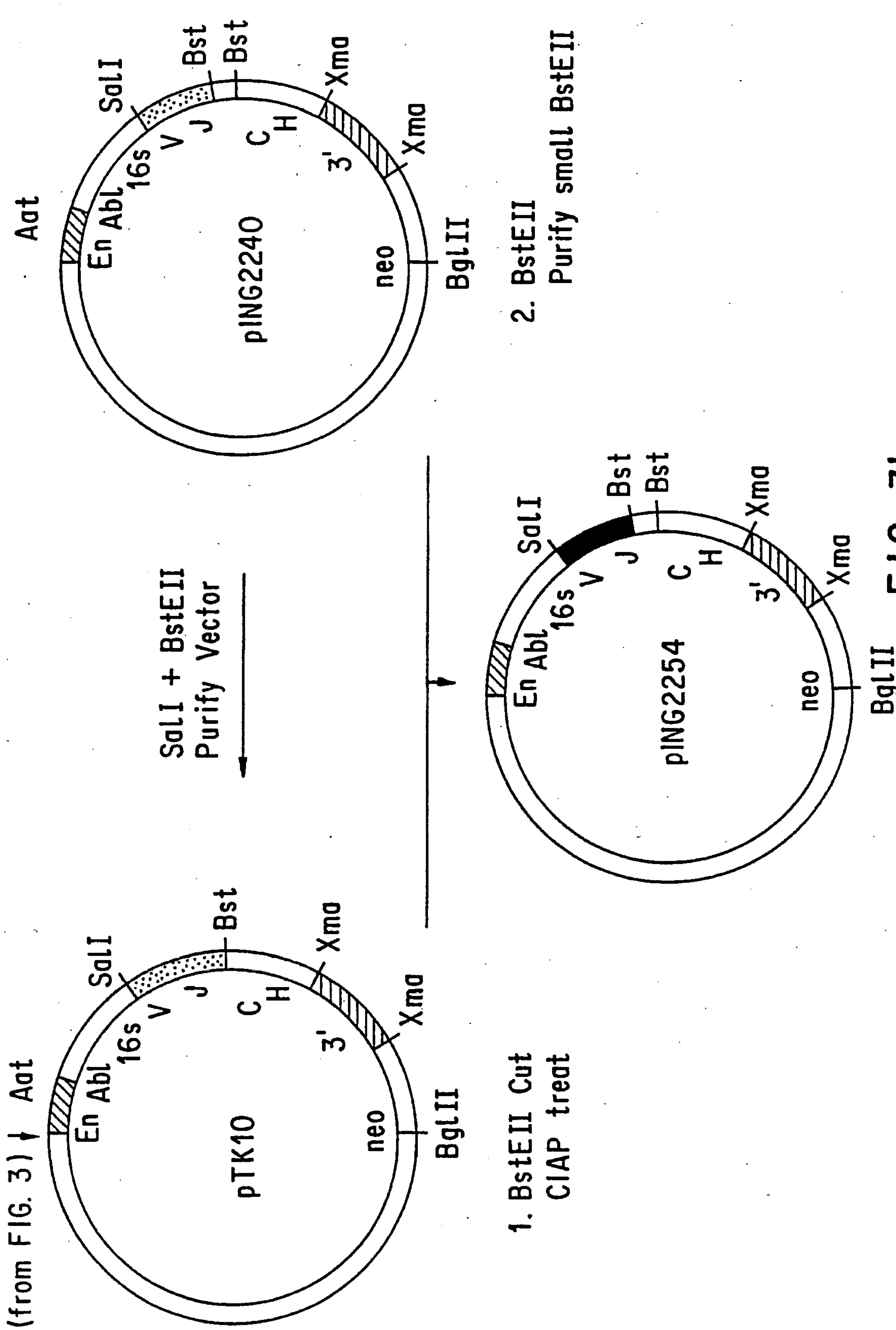

(from FIG. 3)
Aat
En
AbL
16s
V
J
C
H
3'
SalI
Bst
Xma
Xma
neo
BglII
pTK10
1. BstEII Cut
CIAP treat
pING2240
2. BstEII
Purify small BstEII
SalI + BstEII
Purify Vector
pING2254

FIG. 3b

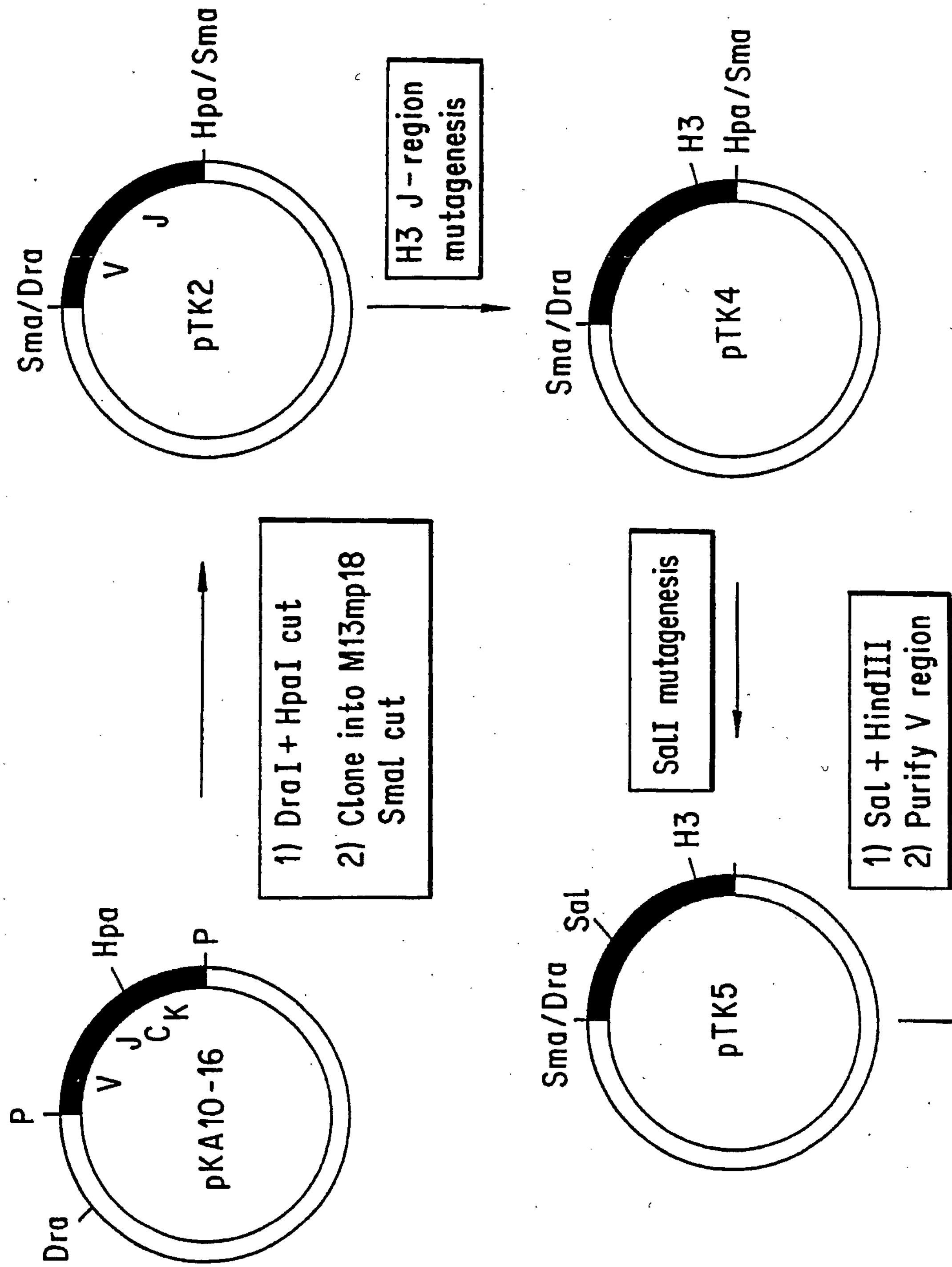


FIG. 4a

(cont. on FIG. 4b)

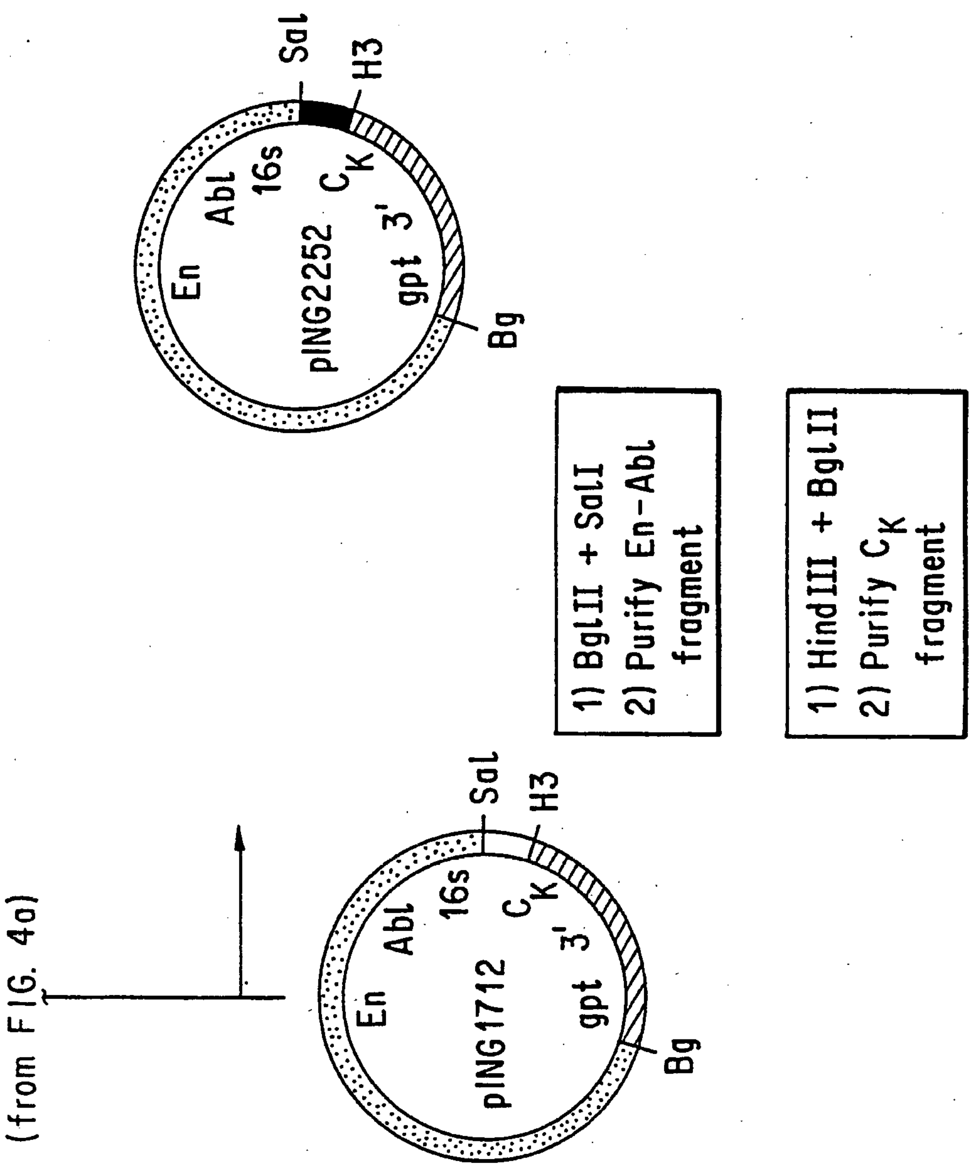
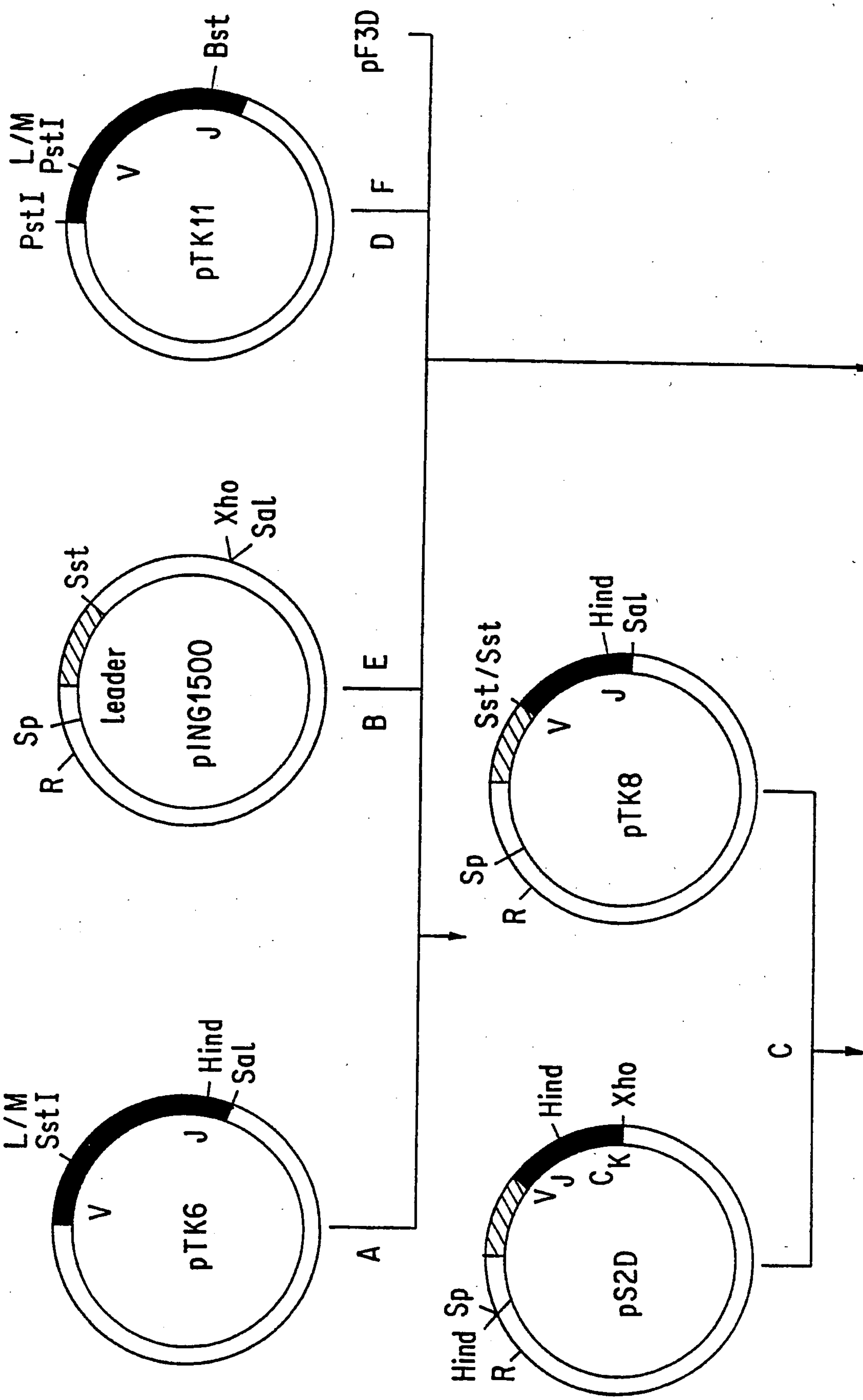

(from FIG. 4a)
pING1712
En
AbL
16s
C_K
3'
gpt
SaL
H3
Bg
pING2252
En
AbL
16s
C_K
3'
gpt
SaL
H3
Bg
1) BgLII + SaLI
2) Purify En-AbL fragment
1) HindIII + BgLII
2) Purify C_K fragment

FIG. 4b



(cont. on FIG. 5b)

(cont. on FIG. 5b)

FIG. 5a

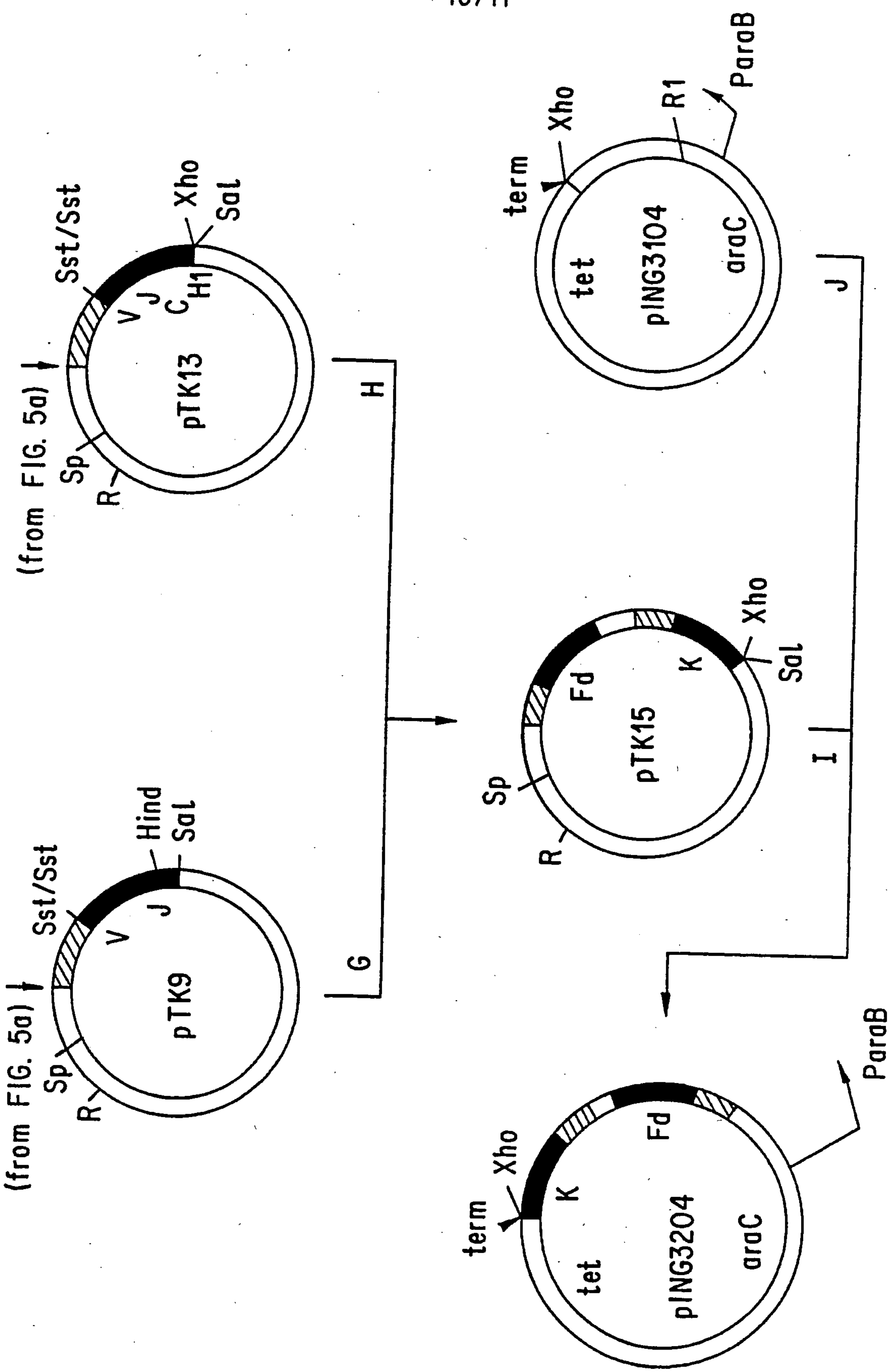

(from FIG. 5a)
Sst/Sst
Xho
Sal
V
J
C_{H1}
pTK13
Sp
R
H
pING3104
ParaB
R1
Xho
term
tet
araC
J
(from FIG. 5a)
Sst/Sst
Hind
Sal
V
J
pTK9
Sp
R
G
pTK15
Fd
K
Xho
Sal
Sp
R
I
pING3204
ParaB
Fd
K
Xho
term
tet
araC

FIG. 5b

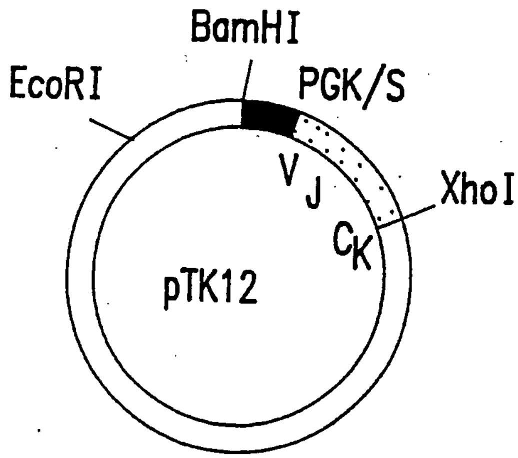


FIG.6a

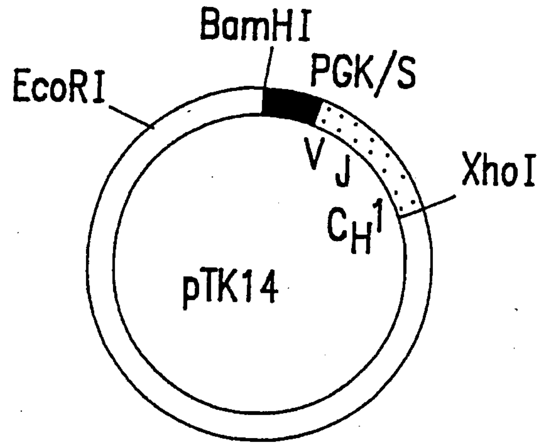


FIG.6b

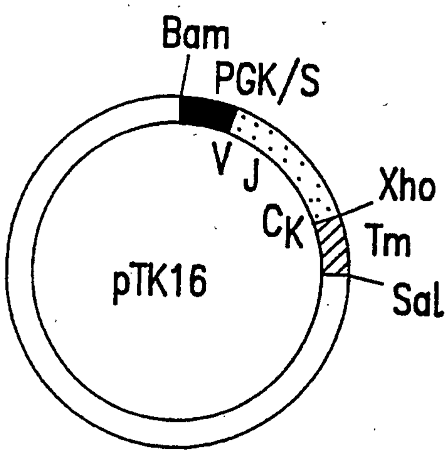


FIG.6c

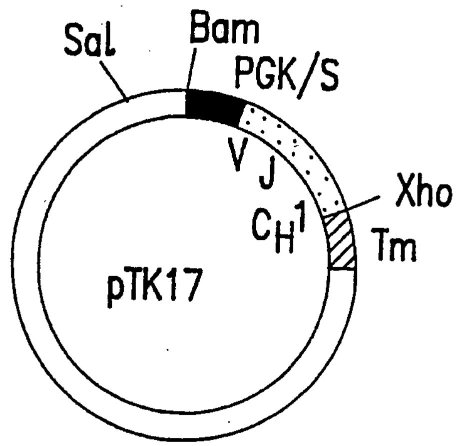


FIG.6d

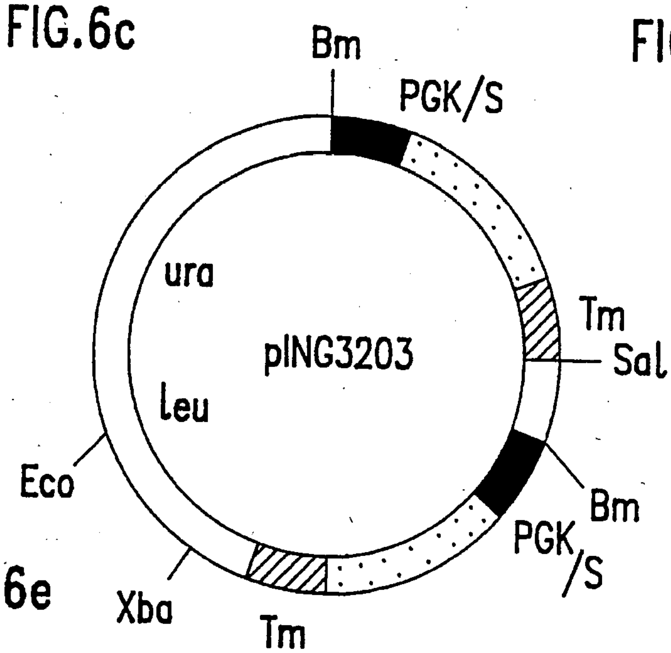


FIG.6e

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/06620

I. CLASSIFICATION F SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C07H 15/12; A61K 1/00; C12N 15/00; G01N 33/563, 33/53; A61K 35/14
U.S. Cl.: 536/27; 514/2; 435/172.3; 436/512, 548; 530/388

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S. Cl.	536/27; 514/2; 435/172.3; 436/512, 548; 530/388

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	The Journal of Immunology, volume 139, No. 10, issued 15 November 1987, **A.Y. LIU, ET AL.**, "Production of a Mouse-Human Chimeric Monoclonal Antibody To CD20 With Potent Fc-Dependent Biologic Activity", pages 3521-3526. See abstract.	14-33
A	Nucleic Acids Research, volume 12, No. 24, issued 1984, **W. KRAMER, ET AL.**, "The Gapped Duplex DNA approach to oligonucleotide-directed mutation construction", pages 9441-9456.	1-33
A	The Journal of Biological Chemistry, volume 261 No. 8, issued 15 March 1986, **XU, ET AL.**, "Transcription Termination and Chromatin Structure of the Active Immunoglobulin k Gene Locus", pages 3838-3845.	1-33
A	Proceedings of The National Academy of Science, volume 82, issued February 1985, **A.N. HOUGHTON, ET AL.**, "Mouse monoclonal IgG3 antibody detecting G_{D3} ganglioside: A phase I trial in patients with malignant melanoma, pages 1242-1246.	1-33

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
08 February 1991	08 MAR 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	John W. Rollins (vsh)

FURTHER INFORMATION CONTINUED FROM THE SEC ND SHEET

A	Proceedings of the National Academy of Science, volume 84, issued May 1987, A.Y. LIU, ET AL., "Chimeric mouse-human IgG1 antibody that can mediate lysis of cancer cells", pages 3439-3443, see abstract and pages 3439-40.	1-33

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers__________, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.